Microbiol. Biotechnol. Lett. (2019), 47(2), 220–233
http://dx.doi.org/10.4014/mbl.1812.12009
pISSN 1598-642X eISSN 2234-7305

MBL
Microbiology and Biotechnology Letters

Optimization of *Streptococcus macedonicus* MBF10-2 Lysate Production in Plant-based Medium by Using Response Surface Methodology

Dini Andyanti[1], Fatin M. Dani[1], Wibowo Mangunwardoyo[2], Muhamad Sahlan[3], and Amarila Malik[1]*

[1]Pharmaceutical Microbiology and Biotechnology Research Group, Faculty of Pharmacy, [2]Department of Biology, Faculty of Mathematics and Natural Science, [3]Bioprocess Research Group, Department of Chemical Engineering, Faculty of Engineering, Universitas Indonesia, UI Campus Depok, Depok 16424, Indonesia

Received: December 17, 2018 / Revised: February 6, 2019 / Accepted: February 8, 2019

Bacterial lysates have become a common ingredient for natural health care. Lactic acid bacteria (LAB) could serve as potential candidates for lysate production: the lactic acids produced by LAB have been utilized for their moisturizing, antimicrobial, and rejuvenating effects, while other substances provide topical benefits and health effects for the skin. Our study aimed to obtain lysate from a LAB *S. macedonicus* MBF 10-2 through an optimized fermentation using the Response Surface Methodology. Strain MBF10-2 was cultivated in a 2L fermenter tank in de Man Rogosa and Sharpe (MRS) medium and in plant-based peptone modified MRS, i.e. Soy-peptone and *Vegitone*. The duration and the medium composition (dextrose and soy peptone or proteose peptone) were adjusted to obtain an optimum production of cell lysate. Central Composite Design was employed for Design Expert 7.0.0 by adjusting 3 factors: dextrose (1%, 1.5%, 2%, 2.5%, 3%), soy or proteose peptone (0.5%, 0.75%, 1%, 1.25% and 1.5%), and duration of fermentation (8, 10, 12 14, and 16 h for MRS-Soy peptone and 15, 17, 19, 21, and 23 h for MRS *Vegitone*). Bacteriocin-Like Inhibitor Substance activity of lysate and pH were used as indicators. The optimum condition for lysate production using MRS Soy Peptone and *Vegitone* are as follows: dextrose concentration 2.5%, plant-based peptone 1.25%, while optimum fermentation duration were 11.18 h (MRS Soy Peptone) and 17 h (MRS *Vegitone*) with a starter concentration of 10% at OD_{600nm} 0.2 ± 0.05. However, the standard MRS medium produced better quality lysate compared to MRS plant-based peptones.

Keywords: **Bacterial lysate, bacteriocin, lactic acid bacteria, MRS, response surface methodology, *Streptococcus macedonicus***

Introduction

Recently, lysates of lactic acid bacteria (LAB) carrying a Generally Recognized As Safe classification (GRAS) have been commonly used as a raw material in various health and skin care products. Some studies have reported that the application of some LAB extracts (e.g., *Lactobacillus johnsonii, Lactobacillus casei, Lactobacillus plantarum,* and *Lactobacillus acidophilus*) have antimicrobial and anti-adhesive effects on cutaneous and mucosal surfaces [1]. This antimicrobial effect is thought to occur due to a peptide known as bacteriocin [1–3]. Its biological action may be either bactericidal or bacteriostatic [4, 5] and it is known as Bacteriocin Like Inhibitory Substance for this reason (BLIS) [6]. Additionally, the lactic acid produced by these bacteria has also been shown to inhibit the growth of various dermal pathogens [7], interfere with essential metabolic functions, eliminate cell membrane permeability, and reduce intracellular pH [7]. As one of the α-hydroxy acids, lactic

***Corresponding author**
Tel: +62-21-7270031, Fax: +62-21-7863433
E-mail: amarila.malik@ui.ac.id

acid also has the potential to improve stratum corneum barrier function and enhance the production of ceramide by keratinocytes [7]. Ceramide, one of the main components of the intracellular lipid lamellar layer in the stratum corneum, plays a role in skin defense and maintains skin hydration [8].

Bacterial lysates may have an adjuvant effect and also reinforce the body's natural barriers [9] by modulating the balance of lipid inflammatory mediators. These lysates are made by blending inactivated or killed whole bacteria depending on the application. For vaccine or pharmaceutical applications, bacterial lysates are derived from inactivated pathogenic bacteria that trigger immune surveillance, while in some cosmetic applications, the bacteria used may be commensal or environmental [9]. Cream containing *Bifidobacterium longum* SP lysate was reported to give a significant effect in reducing skin sensitivity and dryness, while improving skin barrier function, compared to placebo [10]. A study on the application of *Vitreoscilla filiformis* lysates in a topical treatment formulation for atopic dermatitis (AD) showed significantly reduced AD-like inflammation in NC/Nga mice [11].

LAB constitute a physiologically related group of facultative anaerobic, nonsporulating, catalase-negative, Gram-positive, low G+C cocci and rods, which share the capacity to ferment sugars primarily into lactic acid via homo- or heterofermentative metabolism [12]. *S. macedonicus* was isolated during a survey of the LAB microbiota of naturally fermented Greek Kasseri cheese [12, 13] and belongs to the thermophilic, homofermentative LAB strains. It is nutritionally fastidious (multiple amino acid auxotrophies, restricted proteolytic system), requiring the addition of appropriate nitrogen sources (yeast extract) to grow in de Man, Rogosa and Sharpe (MRS) medium and to perform well in milk media [12, 14, 15]. Growth of the bacteria is sustained between 25℃ and 45℃ (optimal growth at 42.3℃) and between pH 5.0 and 8.5 (optimal growth at pH 6.4) [14].

In our previous study, *Streptococcus macedonicus* MBF10-2 strain, referred to as BLIS (MBF10-2) with p-type 636 and isolated from a tofu byproduct collected from a home industry in Tangerang, Indonesia, showed a single broad-spectrum bacteriocin and multiple bacteriocin activities. BLIS (MBF10-2) inhibits the growth of *Micrococcus luteus*, *Streptococcus pyogenes* and *Lactococcus lactis*, and also strains of *Leuconostoc mesenteroides* and *Weissella confusa* [16]. The scalable potential of this specific lysate has not been reported yet, although *Streptococcus*, as one of the most prolific bacteriocin-producing genera, has been reported to have some clinical benefits [5, 16–18].

In order to achieve high performance production of bacteriocin, lactic acid, and other substances at commercial scale, optimization of the fermentation conditions is important. The influencing factors could vary with different types of bacteriocin and may be strain-dependent [19]. This current study aims to determine the optimum lysate fermentation production conditions and identify the best medium composition for producing lysate that will yield large amounts of bacteriocin and lactic acid. Two modified MRS media that use plant-based peptones—MRS-Soy peptone and MRS- *Vegitone*—were used in this study and were compared with standard MRS medium. Optimization was carried out using Response Surface Methodology (RSM).

Materials and Methods

Bacterial strains and growth condition

Streptococcus macedonicus MBF 10-2, strain obtained from our previous studies [16, 20] was used as bacterium for lysate production. MBF10-2 was grown anaerobically in each media within anaerobic jars at 37℃. Standard MRS medium [21] and modified mediums MRS-Soy Peptone and MRS-*Vegitone* were used as both inoculum and fermentation mediums. The MRS-Soy peptone medium consisted of 10 g/l soy peptone, 8 g/l LAB-Lemco (Oxoid, UK), 4 g/l yeast extract (Difco, USA), 2 g/l dipotassium hydrogen phosphate, 5 g/l sodium acetate, 2 g/l ammonium citrate, 0.2 g/l magnesium sulfate, 0.05 g/l manganese sulfate, 0.05% (v/v) Tween 80 (Merck, German), and 20 g/l dextrose (Wako, Japan). MRS *Vegitone* was composed of 10 g/l proteolyzed vegetable peptone (Wako), 5 g/l yeast extract (Difco), 2 g/l dipotassium hydrogen phosphate, 5 g/l sodium acetate, 2 g/l ammonium citrate, 0.1 g/l magnesium sulfate, 0.05 g/l manganese sulfate, 0.01% (v/v) Tween 80 (Merck, Germany), 3 g/l 2-phenylethyl alcohol, 0.004 g/l bromocresol green, 0.0004 g/l Captan (Wako), and 20 g/l dextrose (Wako). For solid media, Bacto agar (Difco) was added to a final concentration of 15 g/l. Incubation was carried out at 37℃ for 24 h in anaerobic jars using AnaeroGen (Oxoid) [16].

Frozen stock of *Streptococcus macedonicus* MBF 10-2 was stored at 80℃ in MRS Broth with 85% (v/v) of sterile glycerol. To obtain a fresh culture, the strain was cultivated (37℃, 24 h in anaerobic conditions) in the new medium before experimental use.

Leuconostoc mesenteroides TISTR 120 was used as indicator bacteria for bacteriocin activity by disc diffusion method and was grown in MRS medium [22] at 32℃ for 24 h under anaerobic conditions. As a non-lactic acid-producing bacterial indicator, *Bacillus subtilis* ATCC 6633 was grown in Nutrient Agar or Broth at 37℃ for 24 h [23] in aerobic conditions.

Strain confirmation and growth curve

Prior to use, strain confirmation of *S. macedonicus* MBF10-2 was carried out by performing visual inspection of morphology, Gram staining, and 16S rDNA molecular identification. Molecular identification 16S rDNA was determined with PCR and DNA sequencing. For the PCR, a pair of primers, that specifically targets the 16S rRNA gene of LAB, were used: 1 μl LABfw (5'-AGA GTT TGA TYM TGG CTC AG-3') 20 pmol/μl and 1 μl LABrv (5'-CAC CGC TAC ACA TGG AG-3') 20 pmol/μl. PCR mix was done with KAPA HiFi HotStart PCR Kit (Kapa Biosystem, USA), which consisted of 13.5 μl of ddH2O, 5 μl of KAPA HiFi GC buffer (5X), 0.5 μl of 1 U/μl KAPA HiFi HotStart DNA Polymerase, 1 μl KAPA dNTP Mix (10 mM each) and 3 μl of genomic DNA of MBF10-2. PCR reaction was performed as described in a a previous report [20]. Electrophoresis was used for DNA visualization with 1% agarose gel in 1X TAE buffer and using a UV transilluminator. DNA sequencing was used to confirm PCR products followed by analysis with BLAST (http://www.ncbi.nlm.nih.gov/BLAST) for species identification.

To obtain optimum bacterial growth conditions, *S. macedonicus* MBF 10-2 was incubated for 24 h at 37℃ under anaerobic conditions. Absorbance values at 600 nm were taken until stationary phase was reached and each value was preceded by a moderate shaking step [24]. Three independent replicates were carried out for each condition; data were then used to make bacterial growth curves.

Cultivation condition and lactic acid monitoring

The preparation of seed cultures was as follows; a single colony from the agar plate was cultivated at 37℃ for 24 h anaerobically using 7 ml of MRS-Soy Peptone medium and 7 ml of MRS *Vegitone*. Approximately 1 ml of seed culture was transferred into 9 ml of broth medium and was incubated at 37℃ for 24 h anaerobically. This seed culture was subsequently re-transferred into 100 ml of new broth medium and continued anaerobic incubation at 37℃ for 24 h. Culture samples were taken at 0, 6, 12, 18, and 24 h, then bacterial cells were collected by centrifugation at 5000 ×*g* for 30 min at 4℃. Culture pH was measured to monitor lactic acid production against the negative indicator *Bacillus subtilis* ATCC 6633. Furthermore, samples of culture were harvested at 5, 7, 9, 11, and 13 h timepoints and then centrifuged to collect the cells. The cells were washed using sterile ddH_2O before cell lysis.

Production of bacterial lysate

Optimization of cell lysis was done using two methods: first, by a mechanical process using an ultrasonicator and second, by an enzymatic process using lysozyme. An Ultrasonic Homogenizer (Sartorius Stedim, Germany) was used for ultrasonication with the bacterial cells suspended in lysis buffer (750 mM, ε-amino caproic acid, 1 mM PMSF, 50 mM Tris-HCl [pH 7.4]) on ice. Repetition (5x, 10x, and 15x), duration (60 s, 75 s, and 90 s), and the amplitude (25%, 30%, 35%) of the cell lysis conditions were optimized. Repeating 15 duty cycles of 0.5 sec for 75 sec with 15-sec intervals were apparently sufficient enough to lyse the bacterial cells as reported [25]. In the second method, 2 mg/ml lysozyme was used as previously reported [26] followed by 30 min of incubation at 30℃, which was repeated once. In both cases, lysis was confirmed by visual inspection and microscopy.

Lysate from both methods were separated by centrifugation at 10000 ×*g* for 15 min at 4℃ as previously reported [25]. Concentrated bacterial lysates were obtained by filtration with a 10-kDa cutoff Amicon (Merck Millipore, Germany) ultracentrifugal filter. The lysate was freeze-dried and kept at 4℃ to avoid degradation.

BLIS activity assay

BLIS activity assays reference to [16]. The clear zone area observed was measured using calipers. Interval sampling of bacterial culture was done at 5, 7, 9, 11, and

http://dx.doi.org/10.4014/mbl.1812.12009

13 h in triplicate.

Experimental design

We used Design Expert version 7.0.0 for a response surface methodology (RSM) study using central composite design (CCD). We chose three factors as variables or independent variables, namely, the amounts of dextrose (%w/v), plant-based peptone source (proteose peptone (Vegitone) or soy peptone), and fermentation duration (h). Durations of fermentation were 8, 10, 12, 14, and 16 h for MRS-Soy peptone and 15, 17, 19, 21, and 23 h for MRS *Vegitone*. Our two responses as dependent variables were the BLIS activities of *S. macedonicus* MBF 10-2 lysates (in zone of inhibition diameter; mm) and the pH.

Our CCD matrix consisted of 2^3 (8) factorial points with 6 star points and 6 replications at the center points with a total experimental number of 20. The midpoints of dextrose, proteose peptone vegetable, and soy peptone were determined based on the standard percentage used in MRS medium: 2% for dextrose and 1% each for soy and vegetable peptones. The fermentation duration midpoint was determined from the late log phase of the growth curve. Dextrose experimental amounts were 1%, 1.5%, 2%, 2.5%, and 3% while the peptone experimental amounts were 0.5%, 0.75%, 1%, 1.25%, and 1.5%.

Optimization of medium composition and fermentation duration were carried out with a working volume of 50 ml in a tightly-sealed 50 ml Erlenmeyer container to mimic microaerophilic conditions. After fermentation, the bacterial cells were lysed, lysate pH was measured, and BLIS activity was assayed for incorporation into the RSM. Expected bacteriocin activity was shown as an increase in diameter of the inhibitory zone.

The BLIS activity and pH of *S. macedonicus* MBF 10-2 lysates using standard MRS medium was compared to the BLIS activity and pH of lysate from plant-based modified media.

Results

Bacterial strains confirmation

Morphological characteristics were confirmed by visual and microscopic observations as listed in Table 1. *S. macedonicus* MBF 10-2 colonies grown in MRS medium and modified colonies showed similar characteristics. *L. mesenteroides* and *Bacillus subtilis,* as indicator bacteria, showed typical colony formation [27] as presented in Fig. 1. Molecular identification using 16S rDNA polymerase chain reaction (PCR) and sequencing was used to confirm the starter culture as *S. macedonicus* MBF10-2 at 99% homology.

Cultivation of MBF10-2 and lactic acid product monitoring

During fermentation in MRS medium at 37℃ and pH 6.4, *S. macedonicus* MBF 10-2 grew exponentially for about 4 h after a short lag phase (Fig. 2A). The lag phase in modified medium (MRS Soy Peptone and MRS *Vegitone*) was found to be longer than in standard MRS medium (Figs. 2B, 2C).

Lactic acid product synthesized by *S. macedonicus* MBF 10-2 grown in MRS, MRS-Soy peptone, and MRS-*Vegitone* media increased as the pH lowered. All *S. macedonicus* MBF 10-2 cultivation in this study showed a decrease in pH. In comparison, *Bacillus subtilis* controls had no pH changes during the cultivation period (Fig. 2D). This indicates that the *S. macedonicus* grown in all MRS media demonstrated an increase of lactic acid production.

Table 1. Morphological characteristic for confirmation of bacteria used in this study.

Bacterium	Character						
	Visual			Microscopic		Growth condition	
	Color	Size	Shape	Gram	Shape	Oxygen	Temperature
S. macedonicus MBF10-2 (in MRS)	White	Small	Smooth	Positive	Rod	Anaerobic-Facultative	37℃
S. macedonicus MBF10-2 (in MRS-*Vegitone*)	Green	Small	Smooth	Positive	Rod	Anaerobic-Facultative	37℃
S. macedonicus MBF10-2 (in MRS-Soy peptone)	White	Small	Smooth	Positive	Rod	Anaerob-Facultative	37℃
L. mesenteroides TISTR 120	White	Medium	Smooth	Positive	Ovoid coccus	Aerobic	37℃
Bacillus subtilis ATCC 6633	Off white	Large	Rough	Positive	Rod, with spores	Aerobic	37℃

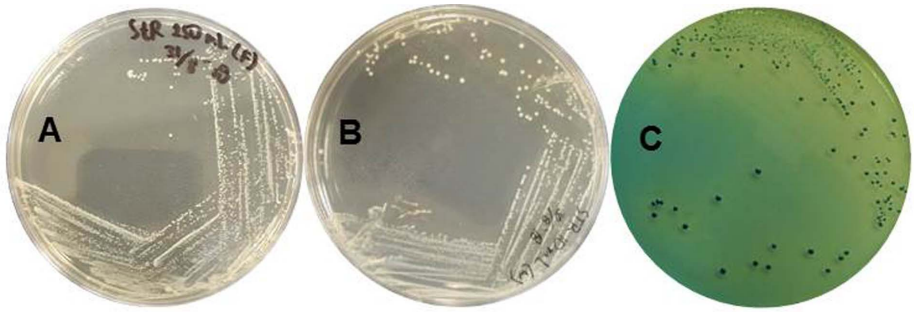

Fig. 1. Colony of *Streptococcus macedonicus* MBF 10-2. (A) MRS Agar, (B) MRS Soy Peptone Agar, (C) MRS *Vegitone* Agar.

Optimum cell lysis condition

Optimization of cell lysis was carried out using an ultrasonicator and lysozyme. Optimization of the cell lysis was performed by modifying the following variables: 1) cycle repetition (5x, 10x, and 15x repetitions); 2) percentage of amplitude (25%, 30%, and 35%); and 3) duration of each repetition (60, 75, and 90 sec). Results showed that the best ultrasonic lysis condition was the 15x replication, 35% amplitude, and 75 sec duration for each repetition, and also by the reduction of cell numbers to 1/8th of the initial cell number. However, after optimization, not all cells were lysed. In addition, the ultrasonication cell lysis process was time-consuming so it was considered ineffective due to the thickness of the

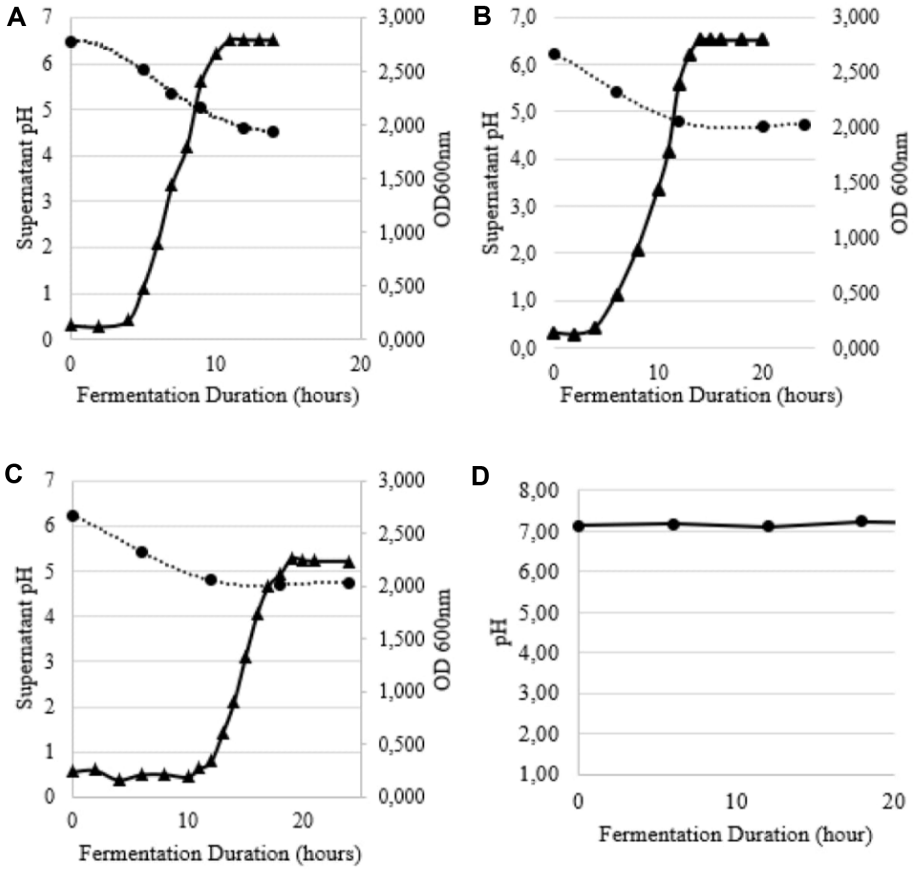

Fig. 2. Bacterial growth curves and pH condition during fermentation. (A) MRS, (B) MRS-Vegitone, (C) MRS-Soy Peptone, (D) pH condition of Bacillus subtilis ATCC 6633 fermentation as negative control; ▲ growth curve, ● pH.

http://dx.doi.org/10.4014/mbl.1812.12009

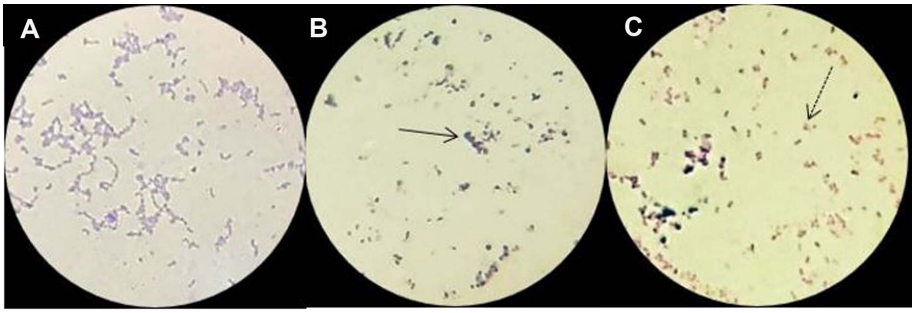

Fig. 3. Cells Lysis of *Streptococcus macedonicus* MBF 10-2. (A) whole cells, (B) destruction cell using ultrasonic, (C) destruction cell using lysozyme. Dense cell represent broken cell is shown by solid arrow; clear intact cell showed by dash arrow.

peptidoglycan wall of *S. macedonicus* MBF 10-2. The subsequent cell separation was therefore carried out enzymatically with lysozyme.

In contrast to mechanical destruction, enzymatic cell destruction using lysozyme resulted in white debris and a clear supernatant. By microscopic observation, it can be assumed that the cell lysis process using lysozyme was more effective than ultrasonication (Fig. 3).

Central point value for CCD during cultivation using RSM

The midpoint of dextrose and soy peptone composition was determined based on the standard percentage used in the MRS medium, i.e. 2% for dextrose and 1% for soy peptone. While the midpoint of fermentation duration was determined based on the growth phases of *S. macedonicus* MBF 10-2 in Medium. The value of CCD presented in Table 2.

Optimizing the fermentation conditions with Design Expert 7.0.0 analysis: Results showed that the probability (P) value < 0.05 of the model treated by using both data responses, i.e. BLIS activity and pH, indicate as significant, and the model was claimed as good with probability value (Prob > F) < 0.05 or significant, while the lack of fit test wass not significant (more than 0.05). From the model, we chose a 50 ml working volume at 37℃ with initial pH 6.79 (Tables 3 and 4).

BLIS activity response: Results obtained by performing Design Expert 7.0.0. using Analysis of variant (ANOVA) of *S. macedonicus* MBF 10-2 lysate in response to BLIS activity of MRS-Soy Peptone culture (Fig. 4A) were analysed and the regression model was given as follows:

$$Y = +6{,}77 + 0{,}26\,A + 0{,}054\,B + 0{,}033\,C + 0{,}15\,AB + 8{,}333E\text{-}003\,AC + 0{,}046\,BC + 0{,}13\,A^2 + 0{,}044\,B^2 + 0{,}017\,C^2 \text{ with } R^2\ 74{,}13\%.$$

Results of BLIS activity of *S. macedonicus* MBF 10-2 lysate in response to BLIS activity of MRS-*Vegitone* culture (Fig. 4B) were analyzed and the regression model was given as follows:

$$Y = +8.93 - 0.15A - 0.056B - 0.10C + 0.54AB - 0.18AC - 0.43BC + 0.018A2 - 0.12B2 - 0.11C2 \text{ with } R^2 = 74{,}60\%.$$

Where A was the percentage of dextrose, B was percentage of soy peptone/proteose peptone vegetable, and C was duration of fermentation.

Table 2. Determination of middle point value of three factors in optimization of medium composition and duration of fermentation with RSM.

Factors	Name	Coded Level				
		-α	-1	0	1	A
A	Dextrose (%)	1	1,5	2	2,5	3
B	Soy Peptone or Proteose Peptone (Vegetable) (%)	0,5	0,75	1	1,25	1,5
C	Duration of Fermentation (Hour) in MRS-Soy Peptone	8	10	12	14	16
	Duration of Fermentation (Hour) in MRS *Vegitone*	15	17	19	21	23

-α: Low Actual, -1: High Actual, 0: Actual, 1: Low Coded, A: High Coded

Table 3. BLIS activity and pH of lysate in MRS-Soy peptone with working volume of 50 ml, Temperature 37 ℃, and initial pH 6.79.

STD	Factor 1 Dextrose (%)	Factor 2 Soy Peptone (%)	Factor 3 Fermentation Duration (h)	Response 2 BLIS Activity (mm)	Response 1 Lysate pH
1	1.50	0.75	10.00	6,60000	7,55
2	2.50	0.75	10.00	7,21667	7,38
3	1.50	1.25	10.00	6,53333	7,33
4	2.50	1.25	10.00	7,38333	7,29
5	1.50	0.75	14.00	6,81667	7,29
6	2.50	0.75	14.00	7,08333	7,20
7	1.50	1.25	14.00	6,55000	7,24
8	2.50	1.25	14.00	7,81667	7,32
9	1.00	1.00	12.00	6,96667	7,11
10	3.00	1.00	12.00	7,56667	7,17
11	2.00	0.50	12.00	6,83333	7,54
12	2.00	1.50	12.00	6,98333	7,12
13	2.00	1.00	8.00	6,80000	7,40
14	2.00	1.00	16.00	6,80000	7,49
15	2.00	1.00	12.00	6,46667	7,39
16	2.00	1.00	12.00	7,16667	7,25
17	2.00	1.00	12.00	6,78333	7,35
18	2.00	1.00	12.00	6,51667	7,31
19	2.00	1.00	12.00	6,86667	7,26
20	2.00	1.00	12.00	6,73333	7,36

Table 4. BLIS activity and pH of lysate in MRS *Vegitone* with working volume of 50 ml, temperature 37 ℃, and Initial pH 6.79.

STD	Factor 1 Dextrose (%)	Factor 2 Soy Peptone (%)	Factor 3 Fermentation Duration (h)	Response 1 BLIS Activity (mm)	Response 2 Lysate pH
1	1,50	0,75	17	9,42	7,32
2	2,50	0,75	17	8,17	7,41
3	1,50	1,25	17	8,62	7,32
4	2,50	1,25	17	9,78	7,27
5	1,50	0,75	21	10,27	7,36
6	2,50	0,75	21	8,57	7,30
7	1,50	1,25	21	8,03	7,19
8	2,50	1,25	21	8,22	7,25
9	1,00	1,00	19	8,87	7,32
10	3,00	1,00	19	8,63	7,33
11	2,00	0,50	19	8,07	7,36
12	2,00	1,50	19	8,50	7,25
13	2,00	1,00	15	8,52	7,29
14	2,00	1,00	23	8,13	7,30
15	2,00	1,00	19	8,43	7,43
16	2,00	1,00	19	9,42	7,42
17	2,00	1,00	19	9,10	7,42
18	2,00	1,00	19	8,73	7,36
19	2,00	1,00	19	8,51	7,40
20	2,00	1,00	19	9,20	7,41

Analysis of variant results for BLIS Activity response of MRS-Soy Peptone and MRS *Vegitone* cultures are listed in Tables 5 and 6. The quadratic model for MRS-Soy peptone and MRS *Vegitone* cultures showed that error did not play an important role at 4.27% and 3.97% chance, respectively. Dextrose is the most significant component in MRS-Soy peptone culture while in MRS-*Vegitone* culture, a significant interaction effect of components were shown. The probability value of Lack of Fit testing in MRS-Soy peptone was 0.5068, while in MRS *Vegitone* it was slightly lower at 0.4145.

Values of pH: Results obtained by performing Design Expert 7.0.0. using ANOVA of *S. macedonicus* MBF 10-2 lysate response to pH value of MRS-Soy Peptone culture (Fig. 5A) were analyzed and the regression model was given as follows:

$$Yi = +7.32 - 6.250E\text{-}003\ A - 0.068\ B - 0.020\ C + 0.038\ AB + 0.025\ AC + 0.048\ BC - 0.44\ A^2 + 3.977E\text{-}0.03\ B^2 + 0.033\ C^2$$

with $R^2 = 0.7338$

Results of lysate pH of *S. macedonicus* MBF 10-2 lysate in response to BLIS activity of MRS-*Vegitone* culture (Fig. 5B) were analyzed and the regression model was given as follows:

$$Yi = +7.40 + 3.672E\text{-}003A - 0.036B - 0.011C - 3.333E - 003AB - 5.833E - 003AC - 1.000E - 002BC - 0.034A2 - 0.027B2 - 0.030C2$$

with $R^2 = 78{,}73\%$.

Analysis of variant results for pH value response of MRS-Soy Peptone and MRS *Vegitone* cultures are shown in Tables 7 and 8. The p-value of quadratic modeling in MRS-Soy peptone and MRS *Vegitone* were 0.048 and

http://dx.doi.org/10.4014/mbl.1812.12009

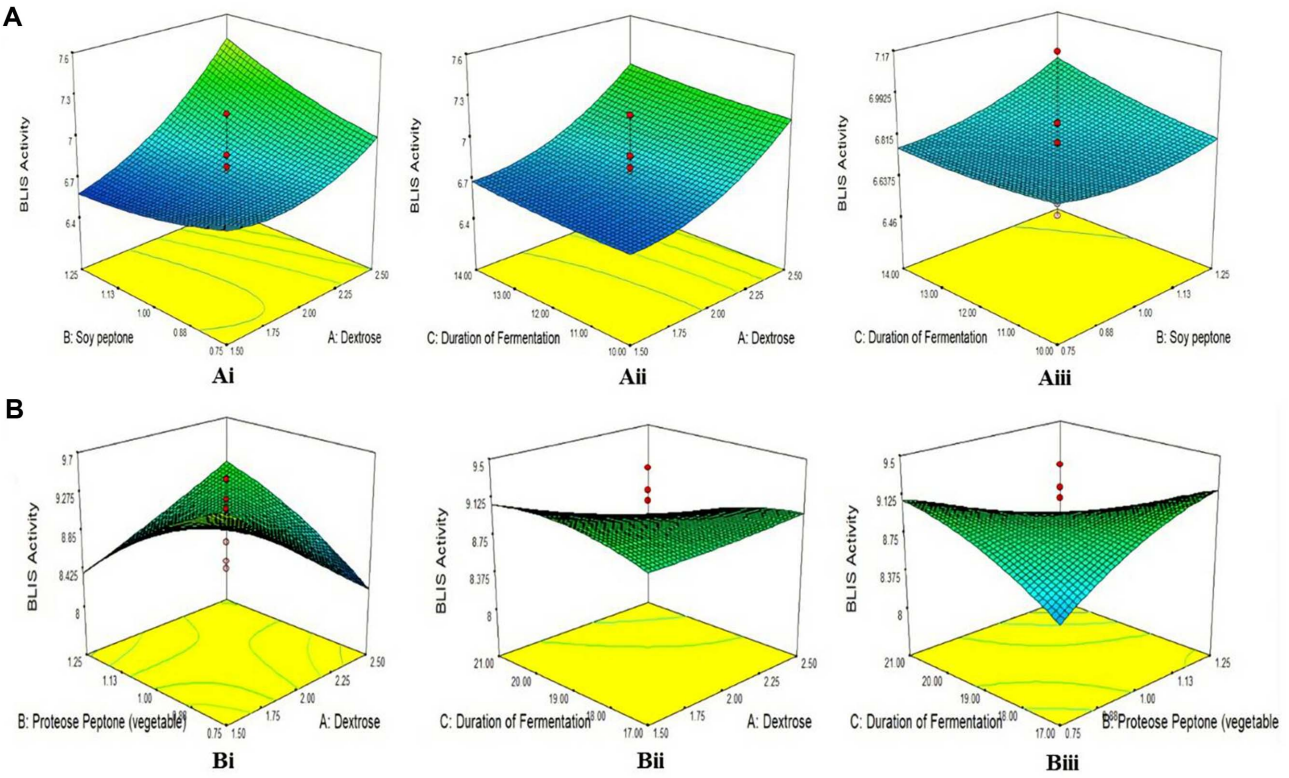

Fig. 4. Interaction between independent factors with BLIS activity presented as a 3 dimenional graphs. (A) dextrose, soy peptone, and fermentation duration; (B) dextrose, proteose peptone vegetable, and fermentation duration. (Ai) Effects of dextrose and soy peptone; (Aii) Effects of dextrose and duration of fermentation; (Aiii) Effects of soy peptone and duration of fermentation; (Bi) Effects of dextrose and proteose peptone vegetable (*Vegitone*); (Bii) Effects of dextrose and duration of fermentation; (Biii) Effects of proteose peptone vegetable (*Vegitone*) and duration of fermentation.

0.0189, respectively. Soy peptone and *Vegitone* were significant components. The probability value of Lack of fit test in MRS-Soy peptone was 0.0867 and in MRS *Vegitone* was 0.0650.

The highest desirability value of the numerical optimization in MRS Soy Peptone was 0.702 with 2.5% of dextrose; 1.25% of soy peptone; and 11.18 h of fermentation duration with a lysate pH of 7.23 and a BLIS activity of 7.38 mm (Tabel 9). The optimum condition for lysate production in MRS *Vegitone* showed an optimal dextrose concentration of 2.5% with optimal proteose peptone vegetable 1.25%, while the optimal fermentation process duration was 17 h with a desirability value of 0.639, a lysate pH of 7.30 and a BLIS activity of 9.76 mm.

Comparison of lysate production in standard MRS and modified MRS

The comparison of optimum medium composition, duration of fermentation and responses are shown in Table 9. BLIS activity of *S. macedonicus* MBF 10-2 lysate from RSM optimization with 2.5% dextrose, 1.25% proteolytic vegetable peptone, and 17 h of fermentation showed an inhibition zone of 9.76 mm, whereas 2.5% dextrose, 1.25% soy peptone, and 11.18 h showed a narrowed zone of 7.38 mm. However, lysates of *S. macedonicus* MBF 10-2 obtained from standard MRS medium showed a larger zone, 10.3 mm (Table 9). It can be concluded that lysate of *S. macedonicus* MBF 10-2 obtained from standard MRS possessed the best BLIS activity. Intracellular lactic acid retention was not a factor as pH of lysates were about the same for standard MRS, MRS *Vegitone* and MRS Soy peptone.

Discussion

To efficiently refine metabolic products such as lactic

Table 5. Results of ANOVA from Design Expert 7.0.0. Software for BLIS activity response in MRS-Soy peptone.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.84	9	0.20	3.18	0.0427	significant
A-Dextrose	1.10	1	1.10	17.20	0.0020	
B-Soy Peptone	0.047	1	0.047	0.73	0.4122	
C-Duration of fermentation	0.018	1	0.018	0.28	0.6100	
AB	0.19	1	0.19	2.97	0.1158	
AC	5.556E-004	1	5.556E-004	8.665E-003	0.9277	
BC	0.017	1	0.017	0.26	0.6198	
A^2	0.45	1	0.45	7.03	0.0242	
B^2	0.049	1	0.049	0.77	0.4007	
C^2	7.468E-003	1	7.468E-003		0.7399	
Residual	0.64	10	0.064	0.12		not significant
Lack of Fit	0.32	5	0.064	0.98	0.5068	
Pure Error	0.32	5	0.065			
Cor Total	2.48	19				
Std. Dev.	0.25		R-Squared		0.7413	
Mean	6.92		Adj R-Squared		0.5085	
C.V. %	3.66		Pred R-Squared		-0.2124	
PRESS	3.00		Adeq Precision		7.252	

acid from bacterial biomass, high cell density is critical for large scale production [29]. To discover the optimum conditions for this fermentation process, we underwent confirmation of the growth curve of MBF10-2. We found that the MBF10-2 lag phases in both kinds of plant-based modified media (MRS *Vegitone* and MRS Soy Peptone) were longer than in standard MRS medium. We presumed this to be an effect of the nitrogen source,

Table 6. Results of ANOVA from Design Expert 7.0.0. Software for BLIS activity response in MRS *Vegitone*.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Model	5.16	9	0.57	3.26	0.0397	significant
A-Dextrose	0.29	1	0.29	1.65	0.2273	
B-Proteose Peptone *(vegetable)*	0.051	1	0.051	0.29	0.6031	
C-Duration of Fermentation	0.17	1	0.17	0.99	0.3436	
AB	2.31	1	2.31	13.16	0.0046	
AC	0.26	1	0.26	1.46	0.2544	
BC	1.45	1	1.45	8.23	0.0167	
A^2	4.499E-003	1	4.499E-003	0.026	0.8760	
B^2	0.37	1	0.37	2.13	0.1753	
C^2	0.31	1	0.31			
Residual	1.76	10	0.18	1.78	0.2120	not significant
Lack of Fit	0.97	5	0.19	1.23	0.4145	
Pure Error	0.79	5	0.16			
Cor Total	6.92	19				
Std. Dev.	0.42		R-Squared		0.7460	
Mean	8.76		Adj R-Squared		0.5174	
C.V. %	4.79		Pred R-Squared		-0.3086	
PRESS	9.05		Adeq Precision		6.875	

http://dx.doi.org/10.4014/mbl.1812.12009

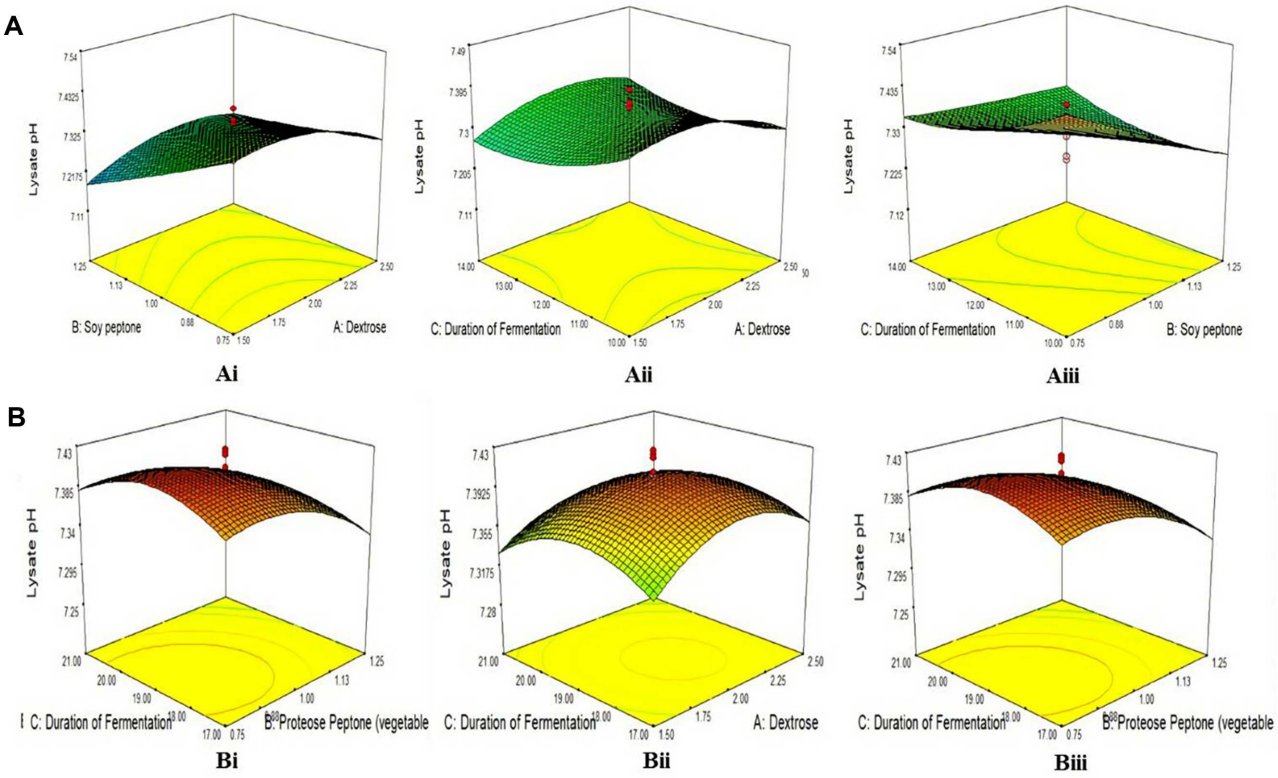

Fig. 5. Interaction between independent factors with pH presented as a 3 dimensional graphs. (A) dextrose, soy peptone, and fermentation duration; (B) dextrose, proteose peptone vegetable, and fermentation duration. (Ai) Effects of dextrose and soy peptone; (Aii) Effects of dextrose and duration of fermentation; (Aiii) Effects of soy peptone and duration of fermentation; (Bi) Effects of dextrose and proteose peptone vegetable (*Vegitone*); (Bii) Effects of dextrose and duration of fermentation; (Biii) Effects of proteose peptone vegetable (*Vegitone*) and duration of fermentation.

namely, the peptone.

The role of the amount of total nitrogen contained in different peptones of the modified media affected the 2 response indicators used in this study, BLIS activity and pH. Total nitrogen in peptone from animal that used in standard MRS medium was ≥10%. While, total nitrogen in plant-based peptone was ≥8%, soy peptone and proteose peptone vegetable (*Vegitone*) were ≥11%, but the latter was later replaced by LAB-Lemco as another nitrogen source. Interestingly, 2-phenylethyl alcohol contained in MRS *Vegitone* may also responsible for prolonging the lag time because of its potential antimicrobial activity (microbial biocontrol) as reported [30] that inhibits mold, yeast, and Gram positive/negative bacteria. It was also reported that 2-phenylethyl alcohol apparently regulates genes involved in peroxisomes, autophagy regulation, the phosphatidylinositol signaling system, and fatty acid synthesis while also inhibiting ribosomes, RNA polymerase, DNA replication, amino acid biosynthesis, aminoacyl-tRNA biosynthesis, and cell cycling [30].

Duration of fermentation is significant as the late log phase is thought to be the highest production phase of lysate active substances, including bacteriocin [14]. In contrast, lysate pH response did not show a significant response as acid is secreted into the milieu during cultivation. *S. macedonicus* MBF 10-2 lysate grown in standard MRS medium showed a slightly lower pH but a greater inhibitory zone than *S. macedonicus* MBF 10-2 lysate grown in modified MRS. Hence, it appeared that lysate from *S. macedonicus* MBF 10-2 grown in standard MRS medium tends to produce more BLIS product.

The LAB lysate produced was intended not only for its BLIS activity and lactic acid production, but also to produce the lysate as a whole ingredient such as acid production, proteolytic and lipolytic activity, citrate

Table 7. Results of ANOVA from Design Expert 7.0.0. Software for lysate pH response in MRS-Soy peptone.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.21	9	0.023	3.06	0.0480	significant
A-Dextrose	6.250E-004	1	6.250E-004	0.082	0.7801	
B-Soy Peptone	0.073	1	0.073	9.59	0.0113	
C-Duration of fermentation	6.400E-003	1	6.400E-003	0.84	0.3803	
AB	0.011	1	0.011	1.48	0.2516	
AC	5.000E-003	1	5.000E-003	0.66	0.4361	
BC	0.018	1	0.018	2.38	0.1543	
A^2	0.048	1	0.048	6.27	0.0312	
B^2	3.977E-004	1	3.977E-004	0.052	0.8236	
C^2	0.027	1	0.027	3.54	0.0891	
Residual	0.076	10	7.598E-003			
Lack of Fit	0.060	5	0.012	3.75	0.0867	not significant
Pure Error	0.016	5	3.200E-003			
Cor Total	0.29	19				
Std. Dev.	0.087		R-Squared		0.7338	
Mean	7.32		Adj R-Squared		0.4942	
C.V. %	1.19		Pred R-Squared		-0.8393	
PRESS	0.52		Adeq Precision		6.222	

metabolism, exopolysaccharide production, antimicrobial activity (macedocin, macedovicin) and biogenic amines production [12, 15, 31].

Bacterial lysates have proven to be effective in the prevention and management of various infections with their immunomodulatory potential. As an example, *Bifidobacterium longum* lysate (BL), strain Bifida ferment lysate, at 5% in total suspension, has an International Nomenclature of Cosmetic Ingredients code for commercial use. It is prepared as an ultrasound-inactivated suspension in aqueous medium and is filter sterilized to remove any remaining whole bacteria. Although an

Table 8. Results of ANOVA from Design Expert 7.0.0. Software for Lysate pH response in MRS *Vegitone*.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.067	9	7.466E-003	4.11	0.0189	significant
A-Dekstrosa	1.842E-004	1	1.842E-004	0.10	0.7566	
B-Proteose Pepton (vegetable)	0.021	1	0.021	11.58	0.0067	
C-Duration of Fermentation	2.025E-003	1	2.025E-003	1.12	0.3157	
AB	8.889E-005	1	8.889E-005	0.049	0.8293	
AC	2.722E-004	1	2.722E-004	0.15	0.7067	
BC	8.000E-004	1	8.000E-004	0.44	0.5218	
A^2	0.016	1	0.016	8.94	0.0136	
B^2	0.019	1	0.019	10.47	0.0089	
C^2	0.023	1	0.023	12.83	0.0050	
Residual	0.018	10	1.815E-003	4.39	0.0650	not significant
Lack of Fit	0.015	5	2.958E-003			
Pure Error	3.365E-003	5	6.730E-004			
Cor Total	0.085	19				
Std. Dev.	0.043		R-Squared		0.7873	
Mean	7.33		Adj R-Squared		0.5959	
C.V. %	0.58		Pred R-Squared		-0.4533	
PRESS	0.12		Adeq Precision		6.030	

http://dx.doi.org/10.4014/mbl.1812.12009

Table 9. Comparison of responses based on optimum fermentation condition.

Response	MRS			MRS *Vegitone*			MRS Soy Peptone		
	2% Dx	1% P	9 h F	2.5% Dx	1.25% PP	17 h F	2.5% Dx	1.25% SP	11 h F
BLIS Activity (mm)					10,3			7,38	
Lysate pH					7,21			7,23	

Dx: Dextrose; P: Peptone; F: Duration of Fermentation; PP: Proteose Peptone Vegetable; SP: Soy Peptone; h: hours.

ultrasonicator is standard in preparation of these lysates, several reports have described preparations of probiotic lysates employing a bead beater and filter sterilization to remove any remaining whole bacteria after growth in anaerobic conditions. These include: *Bifidobacterium longum* ATCC 51870, *Lactobacillus plantarum* ATCC 10241, *Lactobacillus reuteri* ATCC 55730, *Lactobacillus rhamnosus* Goldin and Gorbach [GG] ATCC 53103, and *Lactobacillus fermentum* ATCC 14932. In the case of *Vitreoscilla filiformis* cell lysate, heat can be used by performing steam treatment at 121℃ for 30 min [32]. *V. filiformis* lysate has shown potential application for atopic dermatitis therapy as it reduces pruritus, improves lesions, and stabilizes the skin [32]. In our study, we decided not to lyse bacterial cells via exposure to high temperatures to avoid creation or destruction of therapeutic lysis components.

Bacteriocin activity is first detected in the mid-exponential growth phase and increases rapidly until the end of the exponential growth phase, confirming primary metabolite kinetics [14]. Bacteriocin activity reaches a maximum level at the end of the exponential growth phase and remains constant during the stationary phase, implying that bacteriocin is stable and/or is synthesized de novo, which in line with reports regarding macedocin [14]. With this in mind, we chose the midpoint based on our calculations as the peak of active bacteriocin production.

We chose a quadratic model for both modified mediums because it showed greater R2 values, the *p*-values were significant, and the lack of fit testing was not significant. In this study, BLIS activity assay and pH of lysate using RSM was conducted on a small scale only in order to determine optimum conditions. These two data responses were used to analyze the ratio of dextrose and proteose peptone (vegetable) or soy peptone as the medium composition optimized using RSM. In LAB lysate preparation, lactic acid is an important factor, we regularly monitored the activity of this acid by pH measurement of the culture and lysate.

The optimization of plant-based MRS composition via RSM showed that optimum lysate production is obtained when using 2.5% dextrose and 1.25% soy peptone, but the duration of fermentation varied at 11.18 h and 17 h, for MRS-Soy peptone and MRS *Vegitone*, respectively. Interestingly, the BLIS activity zone of inhibition lysate produced in MRS *Vegitone* was higher than in MRS-Soy peptone whereas the pH of lysates did not significantly differ. However, *S. macedonicus* MBF 10-2 lysate showed a slightly lower pH and much greater BLIS activity zone, when fermented on a standard MRS medium. Hence, MRS *Vegitone* is recommended as a plant-based medium in MBF10-2 lysate production. The use of plant-based medium rather than animal based-materials, especially in strict vegetarian food products [33], is becoming a preference. In addition, Moslems tend to avoid non-halal materials as much as possible. Some reports also described the use of vegetable N-source instead in an attempt to lower the cost of the culture medium for larger scale [33, 34, 36, 37].

Acknowledgments

This work was supported by Research Grant Hibah PDUPT no. 261/UN2.R3.1/HKP.05.00/2018 from the Ministry of Research, Technology and Higher Education Republic of Indonesia to AM. Authors are greatly thanks Dr. Thomas D. Mayers, Assist. Prof. at the Faculty of Medicine, Medical English Communications Center, University of Tsukuba, Japan, for English supervision of this manuscript.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Gueniche A, Benyacoub J, Blum S, Breton L, Castiel I. 2009. Probiotics for skin benefits, pp. 421-439. *In* Nutritional Cosmetics. Elsevier.
2. Lahtinen S. 2012. *Lactic acid bacteria : microbiological and functional aspects*. 4th ed. Boca Raton. FL, CRC Press.
3. Cotter PD, Hill C, Ross RP. 2005. Bacteriocins: developing innate immunity for food. *Nat. Rev. Microbiol*. **3**: 777-788.
4. Sidek NL, Halim M, Tan JS, Abbasiliasi S, Mustafa S, Ariff AB. 2018. Stability of bacteriocin-like inhibitory substance (BLIS) produced by *Pediococcus acidilactici* kp10 at different extreme conditions. *BioMed. Res. Int*. **2018**: 5973484.
5. De Vuyst L, Leroy F. 2007. Bacteriocins from lactic acid bacteria: production, purification, and food applications. *J. Mol. Microbiol. Biotechnol*. **13**: 194-199.
6. Tagg J, Bannister LV. 1979. Fingerprinting β-haemolytic *streptococci* by their production of and sensitivity to bacteriocine-like inhibitors. *J. Med. Microbiol*. **12**: 397-411.
7. Lew LC, Gan CY, Liong MT. 2012. Dermal Bioactives from *Lactobacilli* and *Bifidobacteria*. *Ann. Microbiol*. **63**: 1047-1055.
8. Meckfessel MH, Brandt S. 2014. The structure, function, and importance of ceramides in skin and their use as therapeutic agents in skin-care products. *J. Am. Acad. Dermatol*. **71**: 177-184.
9. Sanchez C, Villemeur MD. 2018. From Pharma to Beauty: The Potential of Bacterial Lysates The Potential of Bacterial Lysates. Available from http://www.lallemandpharma.com/wp-content/uploads/2018/04/EBR-April2018-Bacterial-lysates-potential.pdf. Accessed April 20, 2018.
10. Guéniche A, Bastien P, Ovigne JM, Kermici M, Courchay G, Chevalier V, *et al*. 2010. *Bifidobacterium longum* lysate, a new ingredient for reactive skin. *Exp. Dermatol*. **19**: e1-e8.
11. Volz T, Skabytska Y, Guenova E, Chen KM, Frick JS, Kirschning S, *et al*. 2014. Nonpathogenic bacteria alleviating atopic dermatitis inflammation induce IL-10-producing dendritic cells and regulatory Tr1 cells. *J. Invest. Dermatol*. **134**: 96-104.
12. Georgalaki MD, Van Den Berghe E, Kritikos D, Devreese B, Van Beeumen J, Kalantzopoulos G, *et al*. 2002. Macedocin, a food-grade lantibiotic produced by *Streptococcus macedonicus* ACA-DC 198. *Appl. Environ. Microbiol*. **68**: 5891-903.
13. Vuyst LD, Tsakalidou E. 2008. *Streptococcus macedonicus,* a multifunctional and promising species for dairy fermentations. *Int. Dairy J*. **18**: 476-485.
14. Van den Berghe E, Skourtas G, Tsakalidou E, De Vuyst L. 2006. *Streptococcus macedonicus* ACA-DC 198 produces the lantibiotic, macedocin, at temperature and pH conditions that prevail during cheese manufacture. *Int. J. Food Microbiol*. **107**: 138-147.
15. Georgalaki MD, Sarantinopoulos P, Ferreira ES, De Vuyst L, Kalantzopoulos G, Tsakalidou E. 2000. Biochemical properties of *Streptococcus macedonicus* strains isolated from Greek Kasseri cheese. *J. Appl. Microbiol*. **88**: 817-825.
16. Grazia SE, Sumayyah S, Haiti FS, Sahlan M, Heng NCK, Malik A. 2017. Bacteriocin-like inhibitory substance (BLIS) activity of *Streptococcus macedonicus* MBF10-2 and its synergistic action in combination with antibiotics. *Asian Pac. J. Trop. Med*. **10**: 1140-1145.
17. Lorenz TC. 2012. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *J. Vis. Exp*. (63): e3998.
18. Silva CCG, Silva SPM, Ribeiro SC. 2018. Application of bacteriocins and protective cultures in dairy food preservation. *Front. Microbiol*. **9**: 594.
19. Abbasiliasi S, Tan JS, Tengku Ibrahim TA, Bashokouh F, Ramakrishnan NR, Mustafa S, *et al*. 2017. Fermentation factors influencing the production of bacteriocins by lactic acid bacteria: a review. *RSC Adv*. **7**: 29395-29420.
20. Malik A, Radji M, Kralj S, Dijkhuizen L. 2009. Screening of lactic acid bacteria from Indonesia reveals glucansucrase and fructansucrase genes in two different *Weissella confusa* strains from soya. *FEMS Microbiol. Lett*. **300**: 131-138.
21. De Man J, Rogosa M, Sharpe M. 1960. A Medium for the cultivation of *Lactobacilli*. *J. Appl. Bacteriol*. **23**: 130-135.
22. Pujato SA, Guglielmotti DM, Martínez-García M, Quiberoni A, Mojica FJM. 2017. *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides* bacteriophages: Genomics and cross-species host ranges. *Int. J. Food Microbiol*. **257**: 128-137.
23. Ng HS, Chai CXY, Chow YH, Loh WLC, Yim HS, Tan JS, *et al*. 2018. Direct recovery of *Bacillus subtilis* xylanase from fermentation broth with an alcohol/salt aqueous biphasic system. *J. Biosci. Bioeng*. **125**: 585-589.
24. Kareb O, Champagne CP, Jean J, Gomaa A, Aïder M. 2018. Effect of electro-activated sweet whey on growth of *Bifidobacterium, Lactobacillus,* and *Streptococcus* strains under model growth conditions. *Food Res. Int*. **103**: 316-325.
25. Salzano AM, Novi G, Arioli S, Corona S, Mora D, Scaloni A. 2013. Mono-dimensional blue native-PAGE and bi-dimensional blue native/urea-PAGE or/SDS-PAGE combined with nLC-ESI-LIT-MS/MS unveil membrane protein heteromeric and homomeric complexes in *Streptococcus thermophilus*. *J. Proteomics*. **94**: 240-261.
26. Cappannella E, Benucci I, Lombardelli C, Liburdi K, Bavaro T, Esti M. 2016. Immobilized lysozyme for the continuous lysis of lactic bacteria in wine: Bench-scale fluidized-bed reactor study. *Food Chem*. **210**: 49-55.
27. Dimic G. 2006. Characteristics of the *Leuconostoc mesenteroides subsp. mesenteroides* strains from fresh vegetables. *Acta. Period Technol*. **37**: 3-11.
28. Othman M, Ariff AB, Wasoh H, Kapri MR, Halim M. 2017. Strategies for improving production performance of probiotic *Pediococcus acidilactici* viable cell by overcoming lactic acid inhibition. *AMB Express*. **7**: 215.
29. Liu W, Pang H, Zhang H, Cai Y. 2014. Biodiversity of lactic acid bacteria, pp.103-203. in Lactic Acid Bacteria. *Springer*.
30. Georgalaki M, Papadimitriou K, Anastasiou R, Pot B, Van Driessche G, Devreese B, *et al*. 2013. Macedovicin, the second food-grade lantibiotic produced by *Streptococcus macedonicus* ACA-DC 198. *Food Microbiol*. **33**: 124-130.

http://dx.doi.org/10.4014/mbl.1812.12009

31. Colla LL, Mangano A, Mangano A, Albertin A. 2009. Effects of nonpathogenic gram-negative bacterium *Vitreoscilla filiformis* lysate on atopic dermatitis: a prospective, randomized, double-blind, placebo-controlled clinical study. *Br. J. Dermatol*. **161**: 477-478.
32. Pathak M, Martirosyan D. 2012. Optimization of an effective growth medium for culturing probiotic bacteria for applications in strict vegetarian food products. *FFHDJ*. **2**: 369-378.
33. Bustos G, Moldes AB, Cruz JM, Domínguez JM. 2004. Formulation of low-cost fermentative media for lactic acid production with *Lactobacillus rhamnosus* using vinification lees as nutrients. *J. Agric. Food Chem*. **54**: 801-808.
34. Rodrigues LR, Teixeira JA, Oliveira R. 2006. Low-cost fermentative medium for biosurfactant production by probiotic bacteria. *Biochem. Eng. J*. **32**: 135-142.
35. Zhao Y, Wang Y, Song Z, Shan C, Zhu R, Liu F. 2016. Development of a simple, low-cost and eurytopic medium based on *Pleurotus eryngii* for lactic acid bacteria. *AMB Express*. **6**: 65.